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(54) **BVDV virus-like particles**

(57) The present invention relates to bovine viral diarrhoea virus (BVDV) virus-like particles, a polycistronic RNA and DNA corresponding thereto encoding a polypeptide of BVDV structural proteins that are sufficient to

form BVDV virus-like particles, a viral vector encoding factors and structural proteins for the assembly of BVDV virus-like particles, a vaccine comprising BVDV virus-like particles, a diagnostic kit and methods for preparing BVDV virus-like particles.

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## Description

[0001] The present invention relates to bovine viral diarrhea virus (BVDV) virus-like particles, a polycistronic RNA and DNA corresponding thereto encoding a polyprotein of BVDV structural proteins that are sufficient to form BVDV virus-like particles, a viral vector encoding factors and structural proteins for the assembly of BVDV virus-like particles, a vaccine comprising BVDV virus-like particles, a diagnostic kit and methods for preparing BVDV virus-like particles.

[0002] Bovine viral diarrhea virus (BVDV) is the etiological agent of bovine viral diarrhea in cattle and has a world wide distribution and a prevalence that can be as high as 90%.

[0003] BVDV is a member of the genus pestivirus of the *Flaviviridae* family [Horzinek (1991), Pestiviruses - taxonomic perspectives, Arch. Virology Suppl. 3, 55-65]. BVDV has a positive-stranded RNA genome of approximately 12.5 kilo bases (kb), coding for one open reading frame which can be translated into one large polyprotein [Collet et al. (1988), Proteins encoded by bovine viral diarrhea virus: the genomic organisation of a pestivirus, Virology 165, 200-208]. A BVDV virion consists of a genomic RNA fitted into a nucleocapsid and this capsid is surrounded by an envelope containing glycoproteins.

[0004] The structural proteins, nucleocapsid protein (N) and three envelope glycoproteins (E<sup>ns</sup> or gp48, E1 or gp25 and E2 or gp53) are found in this order close to the N terminal end of the polyprotein. All three glycoproteins are preceded by a signal peptide sequence and are liberated from the polyprotein in the lumen of the endoplasmatic reticulum (ER) by host signal peptidases.

[0005] For a related *Flavivirus*, hepatitis C virus, expression of a genome segment which encodes only the structural proteins is sufficient to form virus-like particles [Baumert et al. (1998), Hepatitis C virus structural proteins assemble into virus-like particles in insect cells, J. Virol. 72, 3827-3836].

[0006] Bovine herpesvirus 1 (BHV-1) is the etiological agent of infectious bovine rhino-tracheitis (IBR) and of infectious pustular vulvovaginitis (IPV) and infectious balanopostitis (IBP). BHV-1 is found in cattle all over the world with high prevalences.

[0007] BHV-1 is a member of the alphaherpesviruses, has a double stranded DNA genome of about 136 kilo base pairs and codes for about 70 genes of which about 30 genes are non essential for the replication of the virus. A spontaneous mutant with a deletion of the non essential glycoprotein E (gE) gene is used in safe and efficacious marker vaccines [Kaashoek et al. (1994), A conventionally attenuated glycoprotein E-negative strain of bovine herpesvirus type 1 is an efficacious and safe vaccine, Vaccine 12, 439-444].

[0008] Vaccines to protect against BVDV induced diseases are based on either inactivated BVDV strains, on subunit vaccines based on isolated BVDV glycoproteins, on attenuated BVDV strains [reviewed by Van Oirschot et al. (1999), Vaccination of cattle against bovine viral diarrhoea, Vet. Microbiol. 64, 169-183], on DNA vaccines [Reddy et al. (1999), Comp Immunol Microbiol Infect Dis. 22, 231-246; Harpin et al. (1999), Vaccination of cattle with a DNA plasmid encoding the bovine viral diarrhoea virus major glycoprotein E2, J. Gen. Virol. 80, 3137-3144], or on vector vaccines. Vaccines based on attenuated BVDV strains may cause immunosuppression and in utero infections [Roth & Kaeberle (1983), Suppression of neutrophil and lymphocyte function induced by a vaccinal strain of bovine viral diarrhea virus with or without administration of ACTH, Am. J. Vet. Res. 44, 2366-2372; Liess et al. (1984), Studies on transplacental transmissibility of a bovine virus diarrhea (BVD) vaccine virus in cattle, Zentrbl. Veterinarmed. Reihe B 31, 669-681] and may be less efficacious in the presence of maternal antibodies.

[0009] Vaccines based on inactivated BVDV or subunit vaccines are less efficacious because the antigens are not produced intracellularly and therefore less efficiently presented to the T cell compartment of the immune system. E.g. two out of three baculovirus BVDV E2 subunit vaccines did not protect against a foetal BVDV infection in a sheep model [Bruschke et al. (1997), A subunit vaccine based on glycoprotein E2 of bovine virus diarrhea virus induces fetal protection in sheep against homologous challenge, Vaccine 15, 1940-1945].

[0010] DNA vaccines and vector vaccines do not have this disadvantage but the presently used DNA and vector vaccines are only expressing part of the structural proteins and fail to form the more immunogenic virus-like particles. E.g. WO 95/12682 describes the expression of only BVDV E2 in the TK locus of a BHV-1 strain [cf. also Kweon et al. (1999), Bovine herpesvirus expressing envelope protein (E2) of bovine viral diarrhea virus as a vaccine candidate, J. Vet. Med. Sci. 61, 395-401].

[0011] BVDV shows antigenic variation and can be divided in several antigenic groups (BVDV IA and IB, and BVDV II). This antigenic variation is mainly based on sequence variations in E2 [van Rijn et al. (1997), Subdivision of the pestivirus genus based on envelope glycoprotein E2, Virology 237, 337-348].

[0012] Vaccines based on E2 of only one BVDV type are more restricted in their cross protection [Bolin and Ridpath (1996), Glycoprotein E2 of bovine viral diarrhea virus expressed in insect cells provides calves limited protection from systemic infection and disease, Arch. Virol. 141, 1463-1477] than vaccines that express also the less variable N, E<sup>ns</sup> and E1 proteins [Elahi et al. (1999), Induction of humoral and cellular immune responses against the nucleocapsid of bovine viral diarrhea virus by an adenovirus vector with an inducible promoter, Virology 261, 1-7].

[0013] The present invention provides BVDV virus-like particles.

[0014] The term "virus-like particles" as used herein refers to particles composed of most or all structural proteins of a virus that have a large part of the structural characteristics in common with their infectious wild type counterparts. However, upon interaction with the host cell, virus-like particles do not produce progeny viruses, because the proper nucleic acid sequences are not present in these particles. In case of BVDV virus-like particles, the nucleocapsids may be empty or may contain irrelevant RNA, but their overall structure is like wild type nucleocapsids and the surrounding envelope contains the same transmembrane glycoproteins E1 and E2 and the same membrane associated protein E<sup>ms</sup>, as are found in wild type BVDV.

[0015] In particular, the virus-like particles according to the present invention comprise the BVDV structural proteins N, E<sup>ms</sup>, E1 and E2. The invention contemplates BVDV virus-like particles which can be derived from various naturally occurring BVDV strains such as BVDV type I A-strains (represented by the NADL strain), BVDV type I B strains (represented by the Osloss strain) and BVDV type II strains (represented by the 890 strain) including the cytopathic strain 87-2552 [Reddy et al. (1995), Antigenic differences between a field isolate and vaccine strains of bovine viral diarrhea virus, J. Clin. Microbiol. 33, 2159-2161] and BVDV type I strain PT810, used as an example in this application. The present invention also contemplates BVDV virus-like particles comprising BVDV structural proteins which are not identical to naturally occurring proteins but contain amino acid substitutions, deletions and/or insertions provided that the mutant structural proteins retain the capability of being assembled into virus-like particles.

[0016] The BVDV structural proteins are derived from a polyprotein which is processed after translation. To prepare the BVDV virus-like particles according to the present invention it is favourable to use host cells which contain the information for synthesising the structural proteins from a DNA template. A cDNA prepared from naturally occurring RNA which codes for the polyprotein of BVDV structural proteins would not work because mRNA transcribed from such a DNA template contains too many splice sites that would be recognised by spliceosomes in the cell nucleus. RNA transcribed from such cDNA would be destroyed before it can be translated.

[0017] The present invention, therefore, provides polycistronic RNA molecules comprising a ribonucleotide sequence which codes for the BVDV structural proteins N, E<sup>ms</sup>, E1 and E2, which RNA is scarcely or not at all being spliced in the cell nucleus within its polyprotein encoding part.

[0018] A preferred embodiment of the RNA molecules according to the present invention is represented by RNA comprising a ribonucleotide sequence which codes for a polyprotein having the amino acid sequence according to SEQ ID NO: 2 and which does not contain strong potential splice sites within its polyprotein encoding part, i.e. no potential splice acceptor sites with a score above -22 and no potential splice donor sites with a score above -13.1.

[0019] A most preferred embodiment of the RNA molecules according to the present invention is represented by a RNA molecule which comprises a ribonucleotide sequence corresponding to the polynucleotide sequence from Nucleotide No. 17 to Nucleotide No. 2710 according to SEQ ID NO: 1.

[0020] The inventive RNA molecules can be obtained from corresponding DNA fragments which are also provided by the present invention.

[0021] The DNA fragments according to the present invention code for the BVDV structural proteins N, E<sup>ms</sup>, E1 and E2, but do not contain strong potential splice sites. Splice sites are recognition sequences in eukaryotic mRNAs that are either exon-intron junctions (splice donor sequences) or intron-exon junctions (splice acceptor sequences) and are used by spliceosomes in the nucleus to remove introns from pre-mRNAs to fuse coding regions that are located on exons together to form a complete open reading frame. BVDV cDNA contains 'accidental' splicing signals that are never used because BVDV RNA normally stays in the cytoplasm. By expressing BVDV cDNA via viral vectors such as BHV1, the RNA will be made in the nucleus and be processed by the spliceosome [Shiu et al. (1997), The presence of RNA splicing signals in the cDNA construct of the E2 gene of classical swine fever virus affected its expression, J. Virol. Methods 69, 223-230].

[0022] To remove most of the (potential) splicing signals from the BVDV cDNA encoding N, E<sup>ms</sup>, E1 and E2, the nucleic acid and protein sequence analysis software system of PC/Gene version 2.32 Jan. 1989 can be used. In this software program the option "splice junctions" which is based on the method of Staden [(1984), Computer methods to locate signals in nucleic acid sequences, Nucl. Acids. Res. 12, 505-519] is appropriate to be used in the present invention.

[0023] A preferred embodiment of the DNA fragments according to the present invention is represented by a DNA comprising the polynucleotide sequence from Nucleotide No. 17 to Nucleotide No. 2710 according to SEQ ID NO: 1.

[0024] The present invention further provides DNA constructs suitable to produce BVDV virus-like particles. In order to allow the BVDV structural proteins to be expressed in host cells the DNA is to be operably linked to cis-regulatory sequences. Such regulatory sequences are capable of binding RNA polymerases in a cell and of initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the cis-regulatory sequences are followed at the 3' terminus by a Kozak consensus sequence and the translation start codon (ATG) of a coding sequence and extend upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the cis-regulatory sequences will be found a transcription initiation site as well as protein binding motifs responsible for the binding of RNA polymerase II complexes.

The choice of such regulatory sequences is obvious to those skilled in the art. Suitable cis-regulatory sequences can be derived from eukaryotic genes, preferably from mammals, or from genes from viruses that infect mammals. Example are the simian virus 40 (SV40) promoter and the cis-regulatory sequences located on the long terminal repeats of retroviruses such as the murine leukemia virus (MuLV) long terminal repeat (MLV-LTR).

**[0025]** In a preferred embodiment of the DNA constructs according to the present invention the DNA encoding the polyprotein of BVDV structural proteins is operably linked to the human cytomegalovirus immediate early 1 promoter.

**[0026]** The DNA constructs according to the present invention preferably further comprise terminator sequences at the 3' terminus of the coding region. mRNA synthesis by RNA polymerase II in eukaryotic cells has a clear starting point, generally at a short distance downstream of the 'TATA-box', but no clear stop signal. The poly(A) signal found upstream of the 3' end of the transcribed region plays an essential role in the cleavage and polyadenylation of the 3' end of mRNAs [Keller (1995), No end yet to messenger RNA 3' processing, Cell 81, 829-832] and is also implicated in termination of transcription, but sequences downstream of this poly(A) signal are thought to play a role in the termination of the mRNA transcription as well. These sequences are implicated in retarding or pausing the transcription complex [Vandenbergh (1991), An apparent pause site in the transcription unit of the rabbit alpha-globin gene, J. Mol. Biol. 220, 255-270]. As efficient termination and polyadenylation confers stability to mRNAs, termination signals -like the one found downstream of the bovine growth hormone gene- are often included in eukaryotic expression vectors.

**[0027]** In a preferred embodiment of the DNA constructs according to the present invention the DNA encoding the polyprotein of BVDV structural proteins is followed by the bovine growth hormone terminator sequence.

**[0028]** From a practical standpoint the use of viral vectors is favourable for the following reasons:

- Viral vectors can express the proteins encoded on inserted gene(s) at high levels;
- the recombinant viruses can be produced to high titres in suitable host cells at lower costs than subunit vaccines;
- upon administration to the host the recombinant viruses express the proteins encoded on inserted gene(s), producing high amounts of these proteins in the host;
- the proteins encoded by the inserted gene(s) are expressed intracellularly and can therefore efficiently be presented by MHC class I molecules to stimulate the cytotoxic T cells of the host;
- all the structural proteins of the BVDV genome can be expressed and form virus-like particles, comparable to modified live virus vaccine, but without the risks normally encountered with such vaccines, like e.g. reversion to wild-type;
- live vaccines are generally more immunogenic than killed vaccines.

**[0029]** The present invention provides a viral vector encoding factors and BVDV structural proteins necessary for the assembly of BVDV virus-like particles.

**[0030]** In a preferred embodiment the viral vector is represented by bovine herpesvirus 1 (BHV-1).

**[0031]** In a further preferred embodiment a BHV-1 vector which contains a deletion within the sequence coding for glycoprotein E (gE) or which completely lacks the genome region coding for gE is used for preparing BVDV virus-like particles. An example for such a BHV-1 vector is Difivac-1 deposited under Accession No. I-1213 with the Institut Pasteur, France. The use of such vector allows the production of a BHV-1/BVDV vaccine with double marker properties. Hosts, in particular cattle, immunised with the BHV-1/BVDV vaccine described herein form antibodies against BHV-1 and BVDV, but not against BHV-1 gE or the BHV-1gI/gE complex and not against the BVDV non structural proteins, in particular NS3 or p80. This allows discrimination of hosts, in particular cattle, infected with wild-type BHV-1 and/or wild-type BVDV from hosts only vaccinated with this BHV-1/BVDV vaccine, by measuring the presence of anti-BHV-1gE or anti-BHV-1gI/gE antibodies and/or anti-BVDV antibodies in their body fluids, in particular nasal fluid samples and serum samples.

**[0032]** A most preferred embodiment of the viral vector according to the present invention is represented by A9-SV-1F9 (synth. BVDV capsid; E<sup>ms</sup>, E1 and E2 in gE locus of Difivac-1) deposited under CNCM accession No. I-2488 with the Collection Nationale de Cultures de Microorganismes, Institut Pasteur (25, Rue du Docteur Roux, F-75724 Paris, France) on June 8, 2000.

**[0033]** The present invention further provides host cells containing the above-mentioned viral vectors. The viral vectors can be introduced into the host cells by infection or transfection. Examples of suitable host cells are embryonic bovine trachea (EBTr) cells or Madin-Darby bovine kidney (MDBK) cells.

**[0034]** The present invention also provides a vaccine for immunising a host against BVDV induced diseases which comprises BVDV virus-like particles and a pharmaceutically acceptable carrier or diluent. Examples of pharmaceutically

acceptable carriers or diluents useful in the present invention include stabilizers such as carbohydrates (e.g. sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein containing agents such as bovine serum or skimmed milk and buffers (e.g. phosphate buffer). Optionally, one or more compounds having adjuvant activity may be added to the vaccine. Suitable adjuvants are for example aluminium hydroxide, phosphate or oxide and oil emulsions such as saponins.

[0035] The useful effective amount to be administered will vary depending on the age, weight, mode of administration and type of pathogen against which vaccination is sought. A suitable dosage can be for example about  $10^3$ - $10^7$  pfu/animal.

[0036] The vaccine according to the present invention can be given inter alia intranasally, intradermally, subcutaneously or intramuscularly.

[0037] A preferred vaccine according to the present invention comprises a BHV-1/BVDV recombinant viral vector which will lead to the intracellular production of BVDV virus-like particles in the vaccinated host and will induce beneficial immune responses to protect against BVDV induced disease. Moreover, such a vaccine will also protect against BHV-1 induced disease and, if BHV-1 lacking gE is used as a vector, hosts vaccinated with such a vaccine can be discriminated from hosts infected either with BHV-1 or with BVDV.

[0038] The present invention further provides a diagnostic kit for detecting the presence and/or absence of anti-BVDV antibodies and, in particular, for detecting the presence and/or absence of anti-BVDV and anti-BHV-1 antibodies in a biological sample such as a biological fluid sample, in particular a nasal fluid sample or a serum sample. These diagnostic tests are used to discriminate between hosts infected with wild type virus and hosts only vaccinated with the BHV-1/BVDV vaccine described herein.

[0039] The anti-BHV-1 and/or anti-BVDV antibodies detected in these diagnostic tests are reacting with viral antigens that are not present in the vaccine, in particular BHV-1gE and the BHV-1 gI/gE complex and the BVDV non structural proteins, in particular NS3 or p80.

[0040] The present invention further provides a method for preparing BVDV virus-like particles comprising

(a) inserting a DNA construct as set forth above into a viral vector encoding factors for the assembly of BVDV virus-like particles,

(b) infecting suitable host cells capable of expressing the polyprotein encoded by the DNA, and

(c) culturing the host cells under appropriate conditions.

[0041] The present invention also provides a method for preparing BVDV virus-like particles comprising

(a) infecting suitable host cells with a viral vector as set forth above, and

(b) culturing the host cells under appropriate conditions.

[0042] The present invention further provides a method for preparing BHV1/BVDV recombinant viruses that encode all structural proteins for the formation of BVDV virus-like particles comprising

(a) inserting a DNA construct as set forth above into the genome of BHV-1,

(b) infecting suitable host cells capable of expressing the polyprotein encoded by the vector, and

(c) culturing the host cells under appropriate conditions.

[0043] Suitable host cells can be infected with the recombinant viruses by adding the supernatant of an infectious culture to a monolayer of these cells, e.g. EBT cells or MDBK cells. For example on a monolayer in a 150 cm<sup>2</sup> tissue culture flask, after removal of the medium, 1 to 3 ml of virus containing (titres ranging from  $10^3$  to  $10^7$  pfu/ml) culture medium can be added. After two hours incubation fresh tissue culture medium can be added up to 30 ml. Two to three days after infection full cytopathogenic effect (cpe) will be visible and up to  $10^8$  pfu/ml can be found in the medium.

## Examples

### Construction of the coding region for BVDV structural proteins

[0044] To determine the amino acid sequences of the structural proteins of a recent BVDV isolate, the 5' half of the

nucleotide sequence of BVDV type I strain PT810, which has recently been isolated in Europe, was established using standard methods.

[0045] The coding regions for the nucleocapsid protein N and the glycoproteins, E<sup>ms</sup>, E1 and E2 were identified by comparing the deduced amino acid sequences with published BVDV sequences. These coding regions contained sequences (splicing signals) which, when expressed by BHV-1 in the nucleus, could be recognized and processed by spliceosomes. Therefore, the sequence was adapted in such a way that most of the (potential) splicing signals were removed while the coding potential remained unaffected. The adapted sequence has been made synthetically and codes for the same amino acids as the originally BVDV sequence with the exception of the first amino acid of the N protein.

[0046] In BVDV strain PT810 the first amino acid of the N protein is a serine but to allow efficient translation of the synthetic coding region, this amino acid was replaced by a methionine and preceded by a Kozak consensus sequence. To end the protein encoded region, a stop codon was inserted behind the putative carboxy-terminal end of the E2 protein, right after its transmembrane region. To allow easy cloning, the sequence recognised by the Stu I restriction enzyme, was added to both sides of the synthetic sequence bringing the total length to 2715 nucleotides. See SEQ ID NO:1. A double stranded DNA fragment has been synthesised and cloned into prokaryotic plasmid Bluescript and propagated in *Escherichia coli* bacteria. See Figure 1.

#### Construction of the BHV-1/BVDV recombination/expression cassette for the gE locus of Difivac-1

[0047] Analysis of the recombination found in the U<sub>S</sub> region of the gE deleted Za strain (which has been renamed Difivac-1 in WO 92/21751) showed the deletion of the glycoprotein E gene and the neighbouring US9 gene and a concomitant duplication inversion of part of the US1.5 gene. This analysis also showed the position of the recombination point [Rijsewijk et al. (1999), Spontaneous BHV-1 recombinants in which the gl/gE/US9 region is replaced by a duplication/inversion of the US1.5/US2 region, Arch. Virol. 144, 1527-1537]. See Figure 2.

[0048] To insert heterologous genes in the genome of the Za strain at or close to the position of the gE deletion, a recombination cassette has been constructed. To this end, a 0.8 kilo base pair Bsa I fragment encoding part of the gl gene and ending 40 bp upstream of the Za recombination point and a 0.7 kilo base pair Bsa I fragment starting 40 bp downstream of the Za recombination point and encoding part of the US1.5 gene, have been cloned in pUC18 in their original orientation. This construct has been named pM400 and has between the recombination fragments a unique Sma I site for the insertion of the expression cassette. See Figure 3.

[0049] In the Sma I site of pM400 a 1.9 kilo base pair Bal I - Acc65 I fragment from plasmid pVR1012 has been cloned with its cis-regulatory sequences in the same orientation as the flanking recombination fragments. These cis-regulatory sequences are the human cytomegalovirus immediate early 1 promoter (hCMVIEp.) and its 5' untranslated leader (5'UT), and the bovine growth hormone terminator sequence (BT). The resulting construct has been named pS205 and has a unique EcoR V site between the promoter region and the terminator region.

[0050] In the EcoR V site of pS205 the 2707 base pair Stu I fragment, encoding the BVDV nucleocapsid protein N and the BVDV glycoproteins, E<sup>ms</sup>, E1 and E2, has been cloned in the same orientation as the promoter/terminator and the flanking recombination sequences. This BHV-1/BVDV recombination/expression cassette has been named pS318. See Figure 3.

#### Construction and isolation of BHV-1 recombinant virus expression the coding region for BVDV structural proteins

[0051] To construct the BHV-1/BVDV recombinant (as described in Figure 4) that expresses the BVDV nucleocapsid protein N and the BVDV glycoproteins, E<sup>ms</sup>, E1 and E2, linearized pS318 plasmid and genomic DNA of the Za strain were cotransfected into bovine cells according to standard methods and 48 hours after cotransfection cells were freeze/thawed to liberate putative recombinant viruses. To isolate these recombinant viruses, the cell debris was pelleted and the supernatant was used to infect bovine cells on a 96 wells plate and to identify BVDV positive wells using an immunostaining procedure with anti-BVDV E2 MAb 166. The virus in the medium of BVDV E2 positive wells was diluted to infect bovine cells on another 96 wells microplate and virus from BVDV E2 positive single plaques was three times plaque purified to obtain a purified recombinant. One of the recombinants obtained this way, was named A9-SV-1F9 and passaged 8 times on MDBK cells to test the stability of the expression on a panel of six different bovine cell lines. See Figure 5. In all bovine cells types tested, even after 8 passages, virtually all plaques were found BVDV E2 positive. In MDBK cells the percentage of BVDV E2 positive plaques was actually determined and found to be after 8 passages still more than 90%.

#### Legends to Sequence Listing and Figures

[0052] SEQ ID NOs: 1 and 2

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The 2715 nucleotides long sequence of the synthetic DNA fragment PT810AM10 which codes for proteins N, E<sup>ms</sup>, E1 and E2 of BVDV strain PT810. To both sides of the DNA fragment the recognition sequence of restriction enzyme Stu I is added and upstream of the start codon (ATG) a Kozak consensus sequences is inserted. The PT810AM10 sequence has been translated using the universal genetic code and the encoded amino acid sequence has been indicated in the three letter code below the nucleotide sequence. All amino acids encoded by the open reading frame are identical to the ones found in BVDV strain PT810, with exception of the first amino acid of the open reading frame. This first amino acid is a serine residue in PT810 and was changed into a methionine residue in PT810AM10. The stop codon at the end of the protein coding region overlaps with the Stu I site at the end of the DNA fragment.

[0053] SEQ ID NOS: 3 and 4

Segment of SEQ ID NO: 1 coding for protein N.

[0054] SEQ ID NOS: 5 and 6

Segment of SEQ ID NO: 1 coding for protein E<sup>ms</sup>.

[0055] SEQ ID NOS: 7 and 8

Segment of SEQ ID NO: 1 coding for protein E1.

[0056] SEQ ID NOS: 9 and 10

Segment of SEQ ID NO: 1 coding for protein E2.

Figure 1

[0057] Structure of plasmid BSM584. The 2715 nucleotides long DNA fragment PT810AM10 has been cloned in prokaryotic plasmid Bluescript. By digesting plasmid BSM584 with restriction enzyme Stu I a 2707 nucleotides long fragment was liberated that was inserted into the BHV-1 recombination/expression cassette pS205. See Figure 3.

Figure 2

[0058] Top: Diagram of the structure of the BHV-1 genome and the recombination found in the Za or Difivac-1 strain that has been used as a vector. Wild type BHV-1 is approximately 136 kilo base pares long and has a Long (L) and a short (S) segment. The short fragment has a unique domain bordered by an inverted repeat indicated by hatched boxes.

[0059] Middle: In unique short (Us) domain 8 open reading frames have been recognised: US1.5, US2, PK, gG, gD, gl gE and US9.

[0060] Bottom: In the natural gE deletion mutant Za or Difivac-1 (Dif.) the gE and US9 genes have been deleted and part of the US1.5 gene has been duplicated instead. The recombination point has been indicated by an arrow. This Difivac-1 mutant has been described in WO 92/21751 and in Arch. Virol. (1999)144, 1527-1537 by Rijsewijk et al.

Figure 3

[0061] Diagram of the recombination/expression cassette plasmid pS318. The 0.8 kilo base pairs Bsa I (B) fragment from the upstream side of the gE locus and the 0.7 kilo base pairs Bsa I fragment from the downstream side of the gE locus, both isolated from the Difivac-1 strain, have been cloned in respectively the Hinc II site and the EcoR I site of prokaryotic plasmid pUC18. To insert the 0.8 kbp Bsa I fragment into the Hinc II site the Bsa I fragment has been made blunt ended and to insert the 0.7 kbp Bsa I fragment into the EcoR I site EcoR I linkers have been added to this fragment using standard methods. The resulting plasmid has been named pM400. In the Sma I site of pM400 a fragment taken from plasmid pVR1012 containing the IE1 promoter/enhancer of the human cytomegalovirus (hCMVIEp.) and the 5' untranslated region of the same promoter (5'UT) and the bovine growth hormone terminator (BT) sequence, has been inserted. The resulting plasmid has been named pS205. Into the unique EcoR V site of pS205 the 2707 bp Stu I fragment from plasmid BSM584 has been inserted. The resulting clone has been named plasmid pS318 and this plasmid has been used in cotransfection experiments to recombine the BVDV genes into the gE locus of Za (Difivac-1). See Figure 4.

Figure 4

[0062] Diagram of the BHV-1/BVDV recombinant A9-SV-1F9 that encodes all structural proteins of BVDV strain PT810. On the top the structure of the BHV-1 genome has been indicated. In the middle the EcoR I fragment that includes the unique short region with the position of all genes and on the bottom the BVDV expression cassette with the 2707 bp long fragment encoding the structural proteins of BVDV stain PT810 have been indicated. The BVDV open reading frame has been preceded by the human cytomegalovirus IE1 promoter/enhancer region (hCMVIEIp.) and the 5' untranslated (5'UT) leader of this promoter and the BVDV open reading frame has been followed by the bovine growth hormone terminator sequence (BT). The cassette has been inserted into the Difivac-1 genome 40 bp upstream

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of the recombination site found in this genome at the gE locus in the same orientation as the surrounding genes.

Figure 5

- 5 **[0063]** IPMA of a monolayer of MDBK cells infected with BHV-1/BVDV recombinant A9-SV-1F9 and stained with anti-BVDV E2 MAb 166 four days after infection. The infected cells form a round plaque that is stained by the antibody while the surrounding cells are not stained.

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## SEQUENCE LISTING

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<223> Kozak consensus sequence

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1 5 10

cag aaa ccg gac cgc gtt gaa aaa ggg cgc atg aaa ata acg cct aaa 100  
Gln Lys Pro Asp Arg Val Glu Lys Gly Arg Met Lys Ile Thr Pro Lys  
15 20 25

gaa act gaa aaa gat tcc cgg acc aaa cca cct gat gct acg atc gtc 148  
Glu Thr Glu Lys Asp Ser Arg Thr Lys Pro Pro Asp Ala Thr Ile Val  
30 35 40

gtc gac ggc gtc aaa tac caa gtc aaa aaa aaa ggc aaa gtc aaa tcc 196  
Val Asp Gly Val Lys Tyr Gln Val Lys Lys Lys Gly Lys Val Lys Ser  
45 50 55 60

aaa aac acc caa gat ggg ctc tac cac aat aaa aat aaa cca caa gaa 244  
Lys Asn Thr Gln Asp Gly Leu Tyr His Asn Lys Asn Lys Pro Gln Glu  
65 70 75

tca cgc aaa aaa ctg gaa aaa gct cta ttg gct tgg gca ata ttg gct 292  
Ser Arg Lys Lys Leu Glu Lys Ala Leu Leu Ala Trp Ala Ile Leu Ala  
80 85 90

gtt gta tta ttt caa gtc aca atg ggg gaa aac ata aca caa tgg aac 340  
Val Val Leu Phe Gln Val Thr Met Gly Glu Asn Ile Thr Gln Trp Asn  
95 100 105

ttg caa gac aat gga acc gaa ggc gtc caa cgg gct atg ttt gaa cgc 388  
Leu Gln Asp Asn Gly Thr Glu Gly Val Gln Arg Ala Met Phe Glu Arg  
110 115 120

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	ggc gtc aat cgg agc tta cat gga atc tgg ccc gaa aaa atc tgc acc	436
	Gly Val Asn Arg Ser Leu His Gly Ile Trp Pro Glu Lys Ile Cys Thr	
	125 130 135 140	
5	ggc gtc cca tct cat ttg gcc acc gat atg gaa ttg aaa cga att cat	484
	Gly Val Pro Ser His Leu Ala Thr Asp Met Glu Leu Lys Arg Ile His	
	145 150 155	
10	gga atg atg gac gca tct gaa aaa acc aac tat aca tgc tgc cgg ctt	532
	Gly Met Met Asp Ala Ser Glu Lys Thr Asn Tyr Thr Cys Cys Arg Leu	
	160 165 170	
15	caa cga cat gaa ttg aat aaa cat ggc tgg tgc aat tgg tac aat atc	580
	Gln Arg His Glu Trp Asn Lys His Gly Trp Cys Asn Trp Tyr Asn Ile	
	175 180 185	
20	gaa cct tgg att ctg ctt atg aat cgg acc caa gct aac ctc act gaa	628
	Glu Pro Trp Ile Leu Leu Met Asn Arg Thr Gln Ala Asn Leu Thr Glu	
	190 195 200	
25	ggc caa cca caa cgc gaa tgc gcc gtc acc tgc cgc tat gac cgg aat	676
	Gly Gln Pro Gln Arg Glu Cys Ala Val Thr Cys Arg Tyr Asp Arg Asn	
	205 210 215 220	
30	tcc gac ttg aat gtc gtg aca caa gcc cgg gac tct ccg aca cca ctt	724
	Ser Asp Leu Asn Val Val Thr Gln Ala Arg Asp Ser Pro Thr Pro Leu	
	225 230 235	
35	acg gga tgc aaa aaa ggg aaa aac ttc tct ttt tgc ggc atc gtc atc	772
	Thr Gly Cys Lys Lys Gly Lys Asn Phe Ser Phe Ser Gly Ile Val Ile	
	240 245 250	
40	caa ggc cct tgc aat ttt gaa att gct gca tct gac gtc ctc ttc aaa	820
	Gln Gly Pro Cys Asn Phe Glu Ile Ala Ala Ser Asp Val Leu Phe Lys	
	255 260 265	
45	gaa cat gac tgc aca tcc ata ttt caa gat act gct cat tac ctc gtt	868
	Glu His Asp Cys Thr Ser Ile Phe Gln Asp Thr Ala His Tyr Leu Val	
	270 275 280	
50	gat ggg atg act aac tct ttg gag tct gct cga caa gga act gca aaa	916
	Asp Gly Met Thr Asn Ser Leu Glu Ser Ala Arg Gln Gly Thr Ala Lys	
	285 290 295 300	
55	cta aca act tgg ctg ggg cga caa ctt ggg ata ttg ggg aaa aaa ctg	964
	Leu Thr Thr Trp Leu Gly Arg Gln Leu Gly Ile Leu Gly Lys Lys Leu	
	305 310 315	
60	gaa aac aaa tcc aaa aca tgg ttc ggg gct tat gca tct tcc cct tac	1012
	Glu Asn Lys Ser Lys Thr Trp Phe Gly Ala Tyr Ala Ser Ser Pro Tyr	
	320 325 330	
65	tgc gat gtt gaa cga aaa ctt ggc tac atc tgg ttt aca aaa aat tgc	1060
	Cys Asp Val Glu Arg Lys Leu Gly Tyr Ile Trp Phe Thr Lys Asn Cys	
	335 340 345	
70	acc cct gcc tgc ctc ccc aaa aac aca aaa att gtt gga cct ggg aaa	1108
	Thr Pro Ala Cys Leu Pro Lys Asn Thr Lys Ile Val Gly Pro Gly Lys	
	350 355 360	
75	ttt gac acc aat gcc gaa gat gga aaa ata tta cat gaa atg ggg ggc	1156
	Phe Asp Thr Asn Ala Glu Asp Gly Lys Ile Leu His Glu Met Gly Gly	
	365 370 375 380	

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5	cac ctt tcg gaa gtt cta tta ctt tca ctt gtt gtt cta tcc gat ttc His Leu Ser Glu Val Leu Leu Leu Ser Leu Val Val Leu Ser Asp Phe 385 390 395	1204
10	gca ccc gaa act gcc tct gcg atg tat ctt gtc cta cat ttt tcc atc Ala Pro Glu Thr Ala Ser Ala Met Tyr Leu Val Leu His Ser Ile 400 405 410	1252
15	cca caa cga cac acc gat gtt ctg gac tgc gat aaa tct caa cta aat Pro Gln Arg His Thr Asp Val Leu Asp Cys Asp Lys Ser Gln Leu Asn 415 420 425	1300
20	cta acc atg ggc gtc aca acc gcc gat gtt ata ccc gga tcc gtc tgg Leu Thr Met Gly Val Thr Thr Ala Asp Val Ile Pro Gly Ser Val Trp 430 435 440	1348
25	aat atg ggc aaa tat gtt tgc ata cga ccc gac tgg tgg cct tat gaa Asn Met Gly Lys Tyr Val Cys Ile Arg Pro Asp Trp Trp Pro Tyr Glu 445 450 455 460	1396
30	acg gct gct gtt ctg gct ttg gaa gaa gtt ggg caa gtt aca cgg atc Thr Ala Ala Val Leu Ala Leu Glu Glu Val Gly Gln Val Thr Arg Ile 465 470 475	1444
35	gtc ttg cgg gca ctc cgc gac ttg aca cgc atc tgg aac gct gcc aca Val Leu Arg Ala Leu Arg Asp Leu Thr Arg Ile Trp Asn Ala Ala Thr 480 485 490	1492
40	acc act gca ttt ctt gtc tgc ctt gtt aaa gtt gtc cgc gga caa gtc Thr Thr Ala Phe Leu Val Cys Leu Val Lys Val Val Arg Gly Gln Val 495 500 505	1540
45	tta caa ggc gtc ata tgg tta ctg cta ata acg ggc gtc caa gga cgc Leu Gln Gly Val Ile Trp Leu Leu Ile Thr Gly Val Gln Gly Arg 510 515 520	1588
50	ctc gat tgc aaa cct gac ttc tca tat gcc att gcc aaa aat gaa aaa Leu Asp Cys Lys Pro Asp Phe Ser Tyr Ala Ile Ala Lys Asn Glu Lys 525 530 535 540	1636
55	att gga cca ctg ggg gct gaa gga ctt act acc act tgg tat gaa tac Ile Gly Pro Leu Gly Ala Glu Gly Leu Thr Thr Thr Trp Tyr Glu Tyr 545 550 555	1684
60	tct gat ggg atg caa ctt tcc gac act atg gtt gaa gct cga tgc aaa Ser Asp Gly Met Gln Leu Ser Asp Thr Met Val Glu Ala Arg Cys Lys 560 565 570	1732
65	gat ggg gaa ttt aca ttc atc caa aaa tgc aaa acg gaa acc cga tat Asp Gly Glu Phe Thr Phe Ile Gln Lys Cys Lys Thr Glu Thr Arg Tyr 575 580 585	1780
70	ctg gcc acc ttg cac aca cgg gcc tta ccg aca tct gtc gtt ttt gaa Leu Ala Thr Leu His Thr Arg Ala Leu Pro Thr Ser Val Val Phe Glu 590 595 600	1828
75	aaa ctt ttt gat gga aat aaa ttg gcg gac atc gtt gaa atg gat gac Lys Leu Phe Asp Gly Asn Lys Leu Ala Asp Ile Val Glu Met Asp Asp 605 610 615 620	1876

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	aac ttc gaa ttt gcg atc tgc ccc tgc gat gca aaa ccc gtc gtc cgc	1924
	Asn Phe Glu Phe Ala Ile Cys Pro Cys Asp Ala Lys Pro Val Val Arg	
	625 630 635	
5	ggg aaa ttt aac aca aca cta cta aat ggg ccc gcc ttc caa atg gtc	1972
	Gly Lys Phe Asn Thr Thr Leu Leu Asn Gly Pro Ala Phe Gln Met Val	
	640 645 650	
10	tgc ccc att gga tgg act gga tct gtc tcc tgc acc cta gcc aat aaa	2020
	Cys Pro Ile Gly Trp Thr Gly Ser Val Ser Cys Thr Leu Ala Asn Lys	
	655 660 665	
	gac acc ctc gat acg gcc gtc gtc cgg aca tat aaa cgc gtt tcc cca	2068
	Asp Thr Leu Asp Thr Ala Val Val Arg Thr Tyr Lys Arg Val Ser Pro	
	670 675 680	
15	ttc cct aat cgg caa gga tgc gtt act caa aaa ctt ctc ggg gaa gat	2116
	Phe Pro Asn Arg Gln Gly Cys Val Thr Gln Lys Leu Leu Gly Glu Asp	
	685 690 695 700	
20	ctt tat gat tgc atc ttg ggc gga aac tgg act tgc atc gaa ggg gaa	2164
	Leu Tyr Asp Cys Ile Leu Gly Gly Asn Trp Thr Cys Ile Glu Gly Glu	
	705 710 715	
	caa cta cga tac act ggg ggc acc att gaa tcc tgc aag tgg tgc ggc	2212
	Gln Leu Arg Tyr Thr Gly Gly Thr Ile Glu Ser Cys Lys Trp Cys Gly	
	720 725 730	
25	tac aaa ttc ttg aaa tcg gaa ggg cta cca cac tat cca att ggc aaa	2260
	Tyr Lys Phe Leu Lys Ser Glu Gly Leu Pro His Tyr Pro Ile Gly Lys	
	735 740 745	
30	tgc cgc tta caa aat gaa act ggc tac cgg ctt gtc gac gac acc tct	2308
	Cys Arg Leu Gln Asn Glu Thr Gly Tyr Arg Leu Val Asp Asp Thr Ser	
	750 755 760	
	tgc aat gtc ggc ggc gtc gca att gtc cca cat gga ctt gtc aaa tgc	2356
	Cys Asn Val Gly Gly Val Ala Ile Val Pro His Gly Leu Val Lys Cys	
	765 770 775 780	
35	aaa att ggg gat acc gtc gtc caa gtc gtc gca atg gac acg aaa ctt	2404
	Lys Ile Gly Asp Thr Val Val Gln Val Val Ala Met Asp Thr Lys Leu	
	785 790 795	
40	gga cct atg cct tgc aaa cca cat gaa ata ata tca tcg gaa gga ccc	2452
	Gly Pro Met Pro Cys Lys Pro His Glu Ile Ile Ser Ser Glu Gly Pro	
	800 805 810	
	gtt gaa aaa acg gca tgc aca ttc aac tat aca cgg acc tta acg aac	2500
	Val Glu Lys Thr Ala Cys Thr Phe Asn Tyr Thr Arg Thr Leu Thr Asn	
	815 820 825	
45	aaa tat ttt gaa ccc cgg gac aat tac ttc caa caa tac atg cta aaa	2548
	Lys Tyr Phe Glu Pro Arg Asp Asn Tyr Phe Gln Gln Tyr Met Leu Lys	
	830 835 840	
50	ggg gac tac caa tat tgg ttt gat ctg gaa gtc tct gac cac cat cgg	2596
	Gly Asp Tyr Gln Tyr Trp Phe Asp Leu Glu Val Ser Asp His His Arg	
	845 850 855 860	
55	gat tac ttt acg gaa ttc cta ctt gtc att gtt gtc gcc ctc ttg ggc	2644
	Asp Tyr Phe Thr Glu Phe Leu Leu Val Ile Val Val Ala Leu Leu Gly	
	865 870 875	

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gga cgc tat gtc ctt tgg cta ctt gtc aca tac atg gtc ctc tcc gaa 2692  
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880 885 890

caa aat gcc tcg gct taggcctt 2715  
Gln Asn Ala Ser Ala  
895

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Asp Ser Arg Thr Lys Pro Pro Asp Ala Thr Ile Val Val Asp Gly Val  
35 40 45

Lys Tyr Gln Val Lys Lys Lys Gly Lys Val Lys Ser Lys Asn Thr Gln  
50 55 60

Asp Gly Leu Tyr His Asn Lys Asn Lys Pro Gln Glu Ser Arg Lys Lys  
65 70 75 80

Leu Glu Lys Ala Leu Leu Ala Trp Ala Ile Leu Ala Val Val Leu Phe  
85 90 95

Gln Val Thr Met Gly Glu Asn Ile Thr Gln Trp Asn Leu Gln Asp Asn  
100 105 110

Gly Thr Glu Gly Val Gln Arg Ala Met Phe Glu Arg Gly Val Asn Arg  
115 120 125

Ser Leu His Gly Ile Trp Pro Glu Lys Ile Cys Thr Gly Val Pro Ser  
130 135 140

His Leu Ala Thr Asp Met Glu Leu Lys Arg Ile His Gly Met Met Asp  
145 150 155 160

Ala Ser Glu Lys Thr Asn Tyr Thr Cys Cys Arg Leu Gln Arg His Glu  
165 170 175

Trp Asn Lys His Gly Trp Cys Asn Trp Tyr Asn Ile Glu Pro Trp Ile  
180 185 190

Leu Leu Met Asn Arg Thr Gln Ala Asn Leu Thr Glu Gly Gln Pro Gln  
195 200 205

Arg Glu Cys Ala Val Thr Cys Arg Tyr Asp Arg Asn Ser Asp Leu Asn  
210 215 220

Val Val Thr Gln Ala Arg Asp Ser Pro Thr Pro Leu Thr Gly Cys Lys  
225 230 235 240

Lys Gly Lys Asn Phe Ser Phe Ser Gly Ile Val Ile Gln Gly Pro Cys  
245 250 255

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	Asn	Phe	Glu	Ile	Ala	Ala	Ser	Asp	Val	Leu	Phe	Lys	Glu	His	Asp	Cys	
				260					265					270			
5	Thr	Ser	Ile	Phe	Gln	Asp	Thr	Ala	His	Tyr	Leu	Val	Asp	Gly	Met	Thr	
			275					280					285				
	Asn	Ser	Leu	Glu	Ser	Ala	Arg	Gln	Gly	Thr	Ala	Lys	Leu	Thr	Thr	Trp	
		290					295					300					
10	Leu	Gly	Arg	Gln	Leu	Gly	Ile	Leu	Gly	Lys	Lys	Leu	Glu	Asn	Lys	Ser	
	305					310					315					320	
	Lys	Thr	Trp	Phe	Gly	Ala	Tyr	Ala	Ser	Ser	Pro	Tyr	Cys	Asp	Val	Glu	
				325						330					335		
15	Arg	Lys	Leu	Gly	Tyr	Ile	Trp	Phe	Thr	Lys	Asn	Cys	Thr	Pro	Ala	Cys	
				340					345					350			
	Leu	Pro	Lys	Asn	Thr	Lys	Ile	Val	Gly	Pro	Gly	Lys	Phe	Asp	Thr	Asn	
			355					360					365				
20	Ala	Glu	Asp	Gly	Lys	Ile	Leu	His	Glu	Met	Gly	Gly	His	Leu	Ser	Glu	
		370					375					380					
	Val	Leu	Leu	Leu	Ser	Leu	Val	Val	Leu	Ser	Asp	Phe	Ala	Pro	Glu	Thr	
	385					390					395					400	
25	Ala	Ser	Ala	Met	Tyr	Leu	Val	Leu	His	Phe	Ser	Ile	Pro	Gln	Arg	His	
				405						410					415		
	Thr	Asp	Val	Leu	Asp	Cys	Asp	Lys	Ser	Gln	Leu	Asn	Leu	Thr	Met	Gly	
			420					425						430			
30	Val	Thr	Thr	Ala	Asp	Val	Ile	Pro	Gly	Ser	Val	Trp	Asn	Met	Gly	Lys	
			435					440					445				
	Tyr	Val	Cys	Ile	Arg	Pro	Asp	Trp	Trp	Pro	Tyr	Glu	Thr	Ala	Ala	Val	
		450					455					460					
35	Leu	Ala	Leu	Glu	Glu	Val	Gly	Gln	Val	Thr	Arg	Ile	Val	Leu	Arg	Ala	
	465					470					475					480	
	Leu	Arg	Asp	Leu	Thr	Arg	Ile	Trp	Asn	Ala	Ala	Thr	Thr	Thr	Ala	Phe	
				485					490						495		
40	Leu	Val	Cys	Leu	Val	Lys	Val	Val	Arg	Gly	Gln	Val	Leu	Gln	Gly	Val	
			500						505					510			
	Ile	Trp	Leu	Leu	Leu	Ile	Thr	Gly	Val	Gln	Gly	Arg	Leu	Asp	Cys	Lys	
		515						520					525				
45	Pro	Asp	Phe	Ser	Tyr	Ala	Ile	Ala	Lys	Asn	Glu	Lys	Ile	Gly	Pro	Leu	
		530					535					540					
	Gly	Ala	Glu	Gly	Leu	Thr	Thr	Thr	Trp	Tyr	Glu	Tyr	Ser	Asp	Gly	Met	
	545					550					555					560	
50	Gln	Leu	Ser	Asp	Thr	Met	Val	Glu	Ala	Arg	Cys	Lys	Asp	Gly	Glu	Phe	
				565						570					575		
55	Thr	Phe	Ile	Gln	Lys	Cys	Lys	Thr	Glu	Thr	Arg	Tyr	Leu	Ala	Thr	Leu	
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His Thr Arg Ala Leu Pro Thr Ser Val Val Phe Glu Lys Leu Phe Asp  
 595 600 605  
 5 Gly Asn Lys Leu Ala Asp Ile Val Glu Met Asp Asp Asn Phe Glu Phe  
 610 615 620  
 Ala Ile Cys Pro Cys Asp Ala Lys Pro Val Val Arg Gly Lys Phe Asn  
 625 630 635 640  
 10 Thr Thr Leu Leu Asn Gly Pro Ala Phe Gln Met Val Cys Pro Ile Gly  
 645 650 655  
 Trp Thr Gly Ser Val Ser Cys Thr Leu Ala Asn Lys Asp Thr Leu Asp  
 660 665 670  
 15 Thr Ala Val Val Arg Thr Tyr Lys Arg Val Ser Pro Phe Pro Asn Arg  
 675 680 685  
 Gln Gly Cys Val Thr Gln Lys Leu Leu Gly Glu Asp Leu Tyr Asp Cys  
 690 695 700  
 20 Ile Leu Gly Gly Asn Trp Thr Cys Ile Glu Gly Glu Gln Leu Arg Tyr  
 705 710 715 720  
 Thr Gly Gly Thr Ile Glu Ser Cys Lys Trp Cys Gly Tyr Lys Phe Leu  
 725 730 735  
 25 Lys Ser Glu Gly Leu Pro His Tyr Pro Ile Gly Lys Cys Arg Leu Gln  
 740 745 750  
 Asn Glu Thr Gly Tyr Arg Leu Val Asp Asp Thr Ser Cys Asn Val Gly  
 755 760 765  
 30 Gly Val Ala Ile Val Pro His Gly Leu Val Lys Cys Lys Ile Gly Asp  
 770 775 780  
 Thr Val Val Gln Val Val Ala Met Asp Thr Lys Leu Gly Pro Met Pro  
 785 790 795 800  
 35 Cys Lys Pro His Glu Ile Ile Ser Ser Glu Gly Pro Val Glu Lys Thr  
 805 810 815  
 Ala Cys Thr Phe Asn Tyr Thr Arg Thr Leu Thr Asn Lys Tyr Phe Glu  
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 40 Pro Arg Asp Asn Tyr Phe Gln Gln Tyr Met Leu Lys Gly Asp Tyr Gln  
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 Tyr Trp Phe Asp Leu Glu Val Ser Asp His His Arg Asp Tyr Phe Thr  
 850 855 860  
 45 Glu Phe Leu Leu Val Ile Val Val Ala Leu Leu Gly Gly Arg Tyr Val  
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 Leu Trp Leu Leu Val Thr Tyr Met Val Leu Ser Glu Gln Asn Ala Ser  
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 55 Ala

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 cgc gtt gaa aaa ggg cgc atg aaa ata acg cct aaa gaa act gaa aaa 96  
 Arg Val Glu Lys Gly Arg Met Lys Ile Thr Pro Lys Glu Thr Glu Lys  
 20 25 30  
 gat tcc cgg acc aaa cca cct gat gct acg atc gtc gtc gac ggc gtc 144  
 Asp Ser Arg Thr Lys Pro Pro Asp Ala Thr Ile Val Val Asp Gly Val  
 35 40 45  
 aaa tac caa gtc aaa aaa aaa ggc aaa gtc aaa tcc aaa aac acc caa 192  
 Lys Tyr Gln Val Lys Lys Lys Gly Lys Val Lys Ser Lys Asn Thr Gln  
 50 55 60  
 gat ggg ctc tac cac aat aaa aat aaa cca caa gaa tca cgc aaa aaa 240  
 Asp Gly Leu Tyr His Asn Lys Asn Lys Pro Gln Glu Ser Arg Lys Lys  
 65 70 75 80  
 ctg gaa aaa gct cta ttg gct tgg gca ata ttg gct gtt gta tta ttt 288  
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 Gln Val Thr Met Gly  
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 35 40 45  
 Lys Tyr Gln Val Lys Lys Lys Gly Lys Val Lys Ser Lys Asn Thr Gln  
 50 55 60  
 Asp Gly Leu Tyr His Asn Lys Asn Lys Pro Gln Glu Ser Arg Lys Lys  
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Gln Val Thr Met Gly  
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1 5 10 15

20

caa cgg gct atg ttt gaa cgc ggc gtc aat cgg agc tta cat gga atc 96  
Gln Arg Ala Met Phe Glu Arg Gly Val Asn Arg Ser Leu His Gly Ile  
20 25 30

25

tgg ccc gaa aaa atc tgc acc ggc gtc cca tct cat ttg gcc acc gat 144  
Trp Pro Glu Lys Ile Cys Thr Gly Val Pro Ser His Leu Ala Thr Asp  
35 40 45

30

atg gaa ttg aaa cga att cat gga atg atg gac gca tct gaa aaa acc 192  
Met Glu Leu Lys Arg Ile His Gly Met Met Asp Ala Ser Glu Lys Thr  
50 55 60

35

aac tat aca tgc tgc cgg ctt caa cga cat gaa tgg aat aaa cat ggc 240  
Asn Tyr Thr Cys Cys Arg Leu Gln Arg His Glu Trp Asn Lys His Gly  
65 70 75 80

40

tgg tgc aat tgg tac aat atc gaa cct tgg att ctg ctt atg aat cgg 288  
Trp Cys Asn Trp Tyr Asn Ile Glu Pro Trp Ile Leu Leu Met Asn Arg  
85 90 95

45

acc caa gct aac ctc act gaa ggc caa cca caa cgc gaa tgc gcc gtc 336  
Thr Gln Ala Asn Leu Thr Glu Gly Gln Pro Gln Arg Glu Cys Ala Val  
100 105 110

50

acc tgc cgc tat gac cgg aat tcc gac ttg aat gtc gtg aca caa gcc 384  
Thr Cys Arg Tyr Asp Arg Asn Ser Asp Leu Asn Val Val Thr Gln Ala  
115 120 125

55

cgg gac tct ccg aca cca ctt acg gga tgc aaa aaa ggg aaa aac ttc 432  
Arg Asp Ser Pro Thr Pro Leu Thr Gly Cys Lys Lys Gly Lys Asn Phe  
130 135 140

tct ttt tgc ggc atc gtc atc caa ggc cct tgc aat ttt gaa att gct 480  
Ser Phe Ser Gly Ile Val Ile Gln Gly Pro Cys Asn Phe Glu Ile Ala  
145 150 155 160

gca tct gac gtc ctc ttc aaa gaa cat gac tgc aca tcc ata ttt caa 528  
Ala Ser Asp Val Leu Phe Lys Glu His Asp Cys Thr Ser Ile Phe Gln  
165 170 175

gat act gct cat tac ctc gtt gat ggg atg act aac tct ttg gag tct 576  
Asp Thr Ala His Tyr Leu Val Asp Gly Met Thr Asn Ser Leu Glu Ser

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	ggg ata ttg ggg aaa aaa ctg gaa aac aaa tcc aaa aca tgg ttc ggg Gly Ile Leu Gly Lys Lys Leu Glu Asn Lys Ser Lys Thr Trp Phe Gly 210 215 220			672
10	gct tat gca Ala Tyr Ala 225			681
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	Met Glu Leu Lys Arg Ile His Gly Met Met Asp Ala Ser Glu Lys Thr 50 55 60			
30	Asn Tyr Thr Cys Cys Arg Leu Gln Arg His Glu Trp Asn Lys His Gly 65 70 75 80			
	Trp Cys Asn <sup>A</sup> Trp Tyr Asn Ile Glu Pro Trp Ile Leu Leu Met Asn Arg 85 90 95			
35	Thr Gln Ala Asn Leu Thr Glu Gly Gln Pro Gln Arg Glu Cys Ala Val 100 105 110			
	Thr Cys Arg Tyr Asp Arg Asn Ser Asp Leu Asn Val Val Thr Gln Ala 115 120 125			
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## Claims

1. BVDV virus-like particles.
2. BVDV virus-like particles according to claim 1 comprising the BVDV structural proteins N, E<sup>ns</sup>, E1 and E2.
3. Polycistronic RNA molecule comprising a ribonucleotide sequence encoding a polypeptide consisting of the BVDV structural proteins N, E<sup>ns</sup>, E1 and E2, said RNA molecule being not spliced in the cell nucleus within its polypeptide

encoding part.

4. RNA molecule according to claim 3 encoding a polypeptide having the amino acid sequence according to SEQ ID NO: 2 provided that said RNA molecule does not contain strong potential splice sites.
5. RNA molecule according to claim 4 comprising a ribonucleotide sequence corresponding to the polynucleotide sequence from Nucleotide No. 17 to Nucleotide No. 2710 according to SEQ ID NO: 1
6. DNA fragment corresponding to the RNA molecule according to any of claims 3 to 5.
7. DNA fragment according to claim 6 comprising the polynucleotide sequence from Nucleotide No. 17 to Nucleotide No. 2710 according to SEQ ID NO: 1.
8. DNA construct comprising the DNA according to claim 6 or 7 operably linked to cis-regulatory sequences capable of controlling the expression of the polypeptide encoded by said DNA.
9. DNA construct according to claim 8 further comprising a terminator sequence.
10. DNA construct according to claim 8 or 9 wherein the cis-regulatory sequences are derived from the human cytomegalovirus immediate early 1 promoter and 5' untranslated leader.
11. DNA construct according to claim 9 wherein the terminator sequence is derived from the bovine growth hormone terminator sequence.
12. Viral vector encoding factors for the assembly of BVDV virus-like particles, said viral vector comprising the DNA construct according to any of claims 8 to 11.
13. Viral vector according to claim 12 which is BHV-1 or a BHV-1 deletion mutant.
14. Viral vector according to claim 13 which is Difivac-1 deposited under Accession No. I-1213.
15. Viral vector according to any of claims 12 to 14 wherein said BHV-1 vector carries said DNA construct within the sequence coding for glycoprotein gE or at the position within a mutant BHV-1 vector where the sequence coding for glycoprotein gE is deleted.
16. Viral vector according to claim 15 which is A9-SV-1F9 deposited under the CNCM accession No. I-2488.
17. Host cell containing the vector according to any of claims 12 to 16.
18. Vaccine comprising BVDV virus-like particles according to claim 1 or 2 and a pharmaceutically acceptable carrier or diluent.
19. Vaccine according to claim 18 further comprising BHV-1.
20. Vaccine according to claim 19 wherein said BHV-1 lacks glycoprotein gE.
21. Vaccine according to claim 20 wherein said BHV-1 is Difivac-1 deposited under CNCM accession No. I-1213.
22. Vaccine comprising recombinant viruses encoded by and including the vector according to any of claims 12 to 16 and a pharmaceutically acceptable carrier or diluent.
23. Diagnostic kit containing BVDV virus-like particles according to claim 1 or 2.
24. Diagnostic kit according to claim 23 further containing BVDV NS3 and/or BVDV p80 protein or immunogenic fragments thereof.
25. Diagnostic kit according to claim 23 or 24 further containing BHV-1 gE protein and/or BHV-1 gI/gE protein complex or immunogenic fragments thereof.



26. Method for preparing BVDV virus-like particles comprising

(a) inserting the DNA construct according to any of claims 8 to 11 into a viral vector encoding factors for the assembly of BVDV virus-like particles,

(b) infecting suitable host cells capable of expressing the polyprotein encoded by said DNA, and

(c) culturing said host cells under appropriate conditions.

27. Method for preparing recombinant viruses encoded by and including the vector according to any of claims 12 to 16 comprising

(a) infecting suitable host cells with a viral vector according to 12 to 16,

(b) culturing said host cells under appropriate conditions, and optionally

(c) isolating the recombinant viruses.

28. Method for preparing a vaccine according to claim 22 comprising admixing the recombinant viruses with a pharmaceutically acceptable carrier.

Figure 1

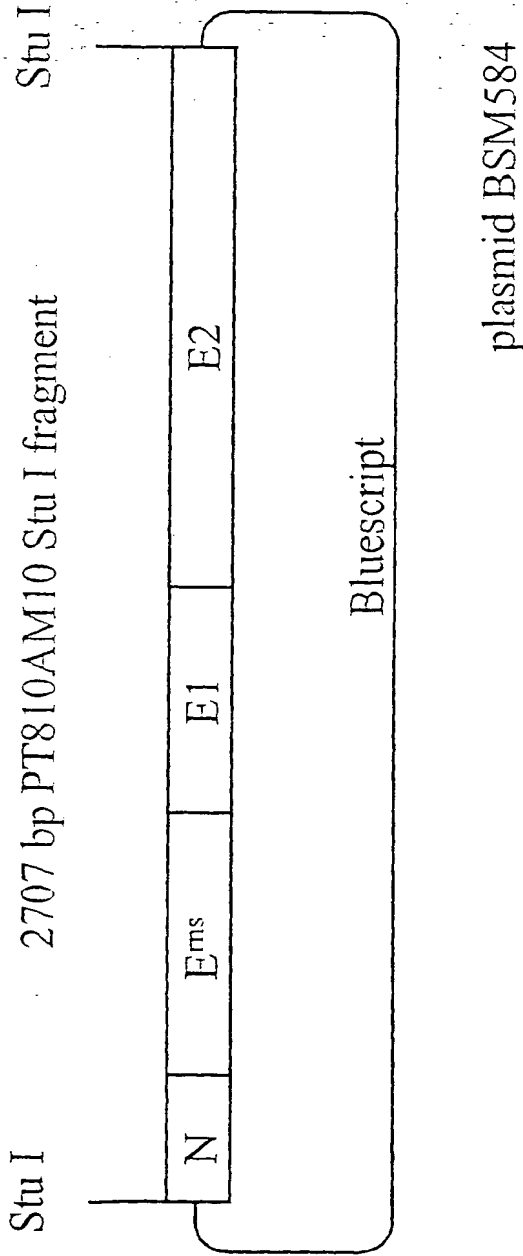


Figure 2

BHV-1

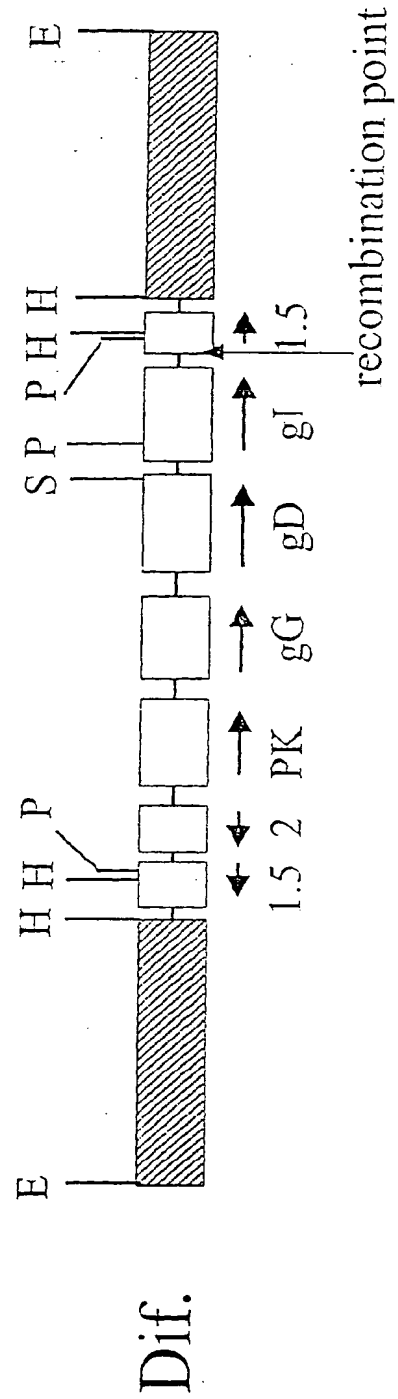
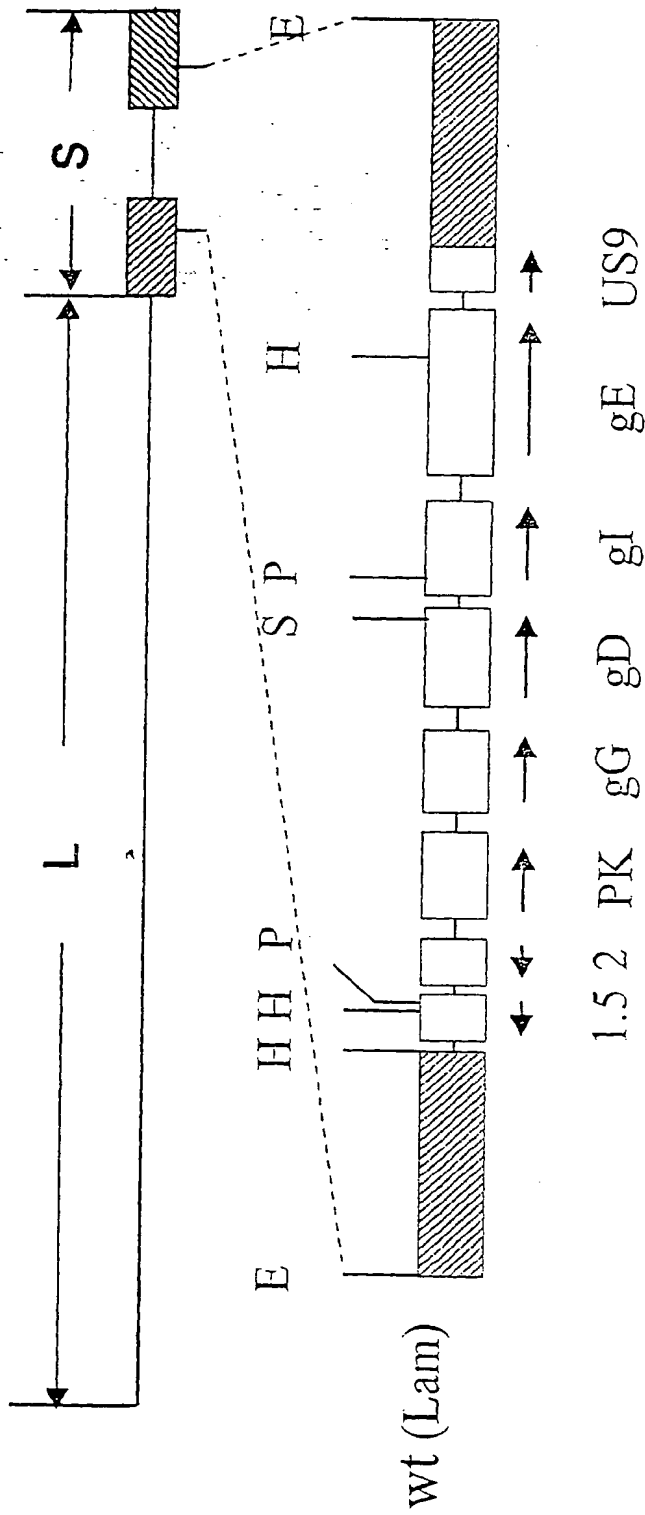


Figure 3

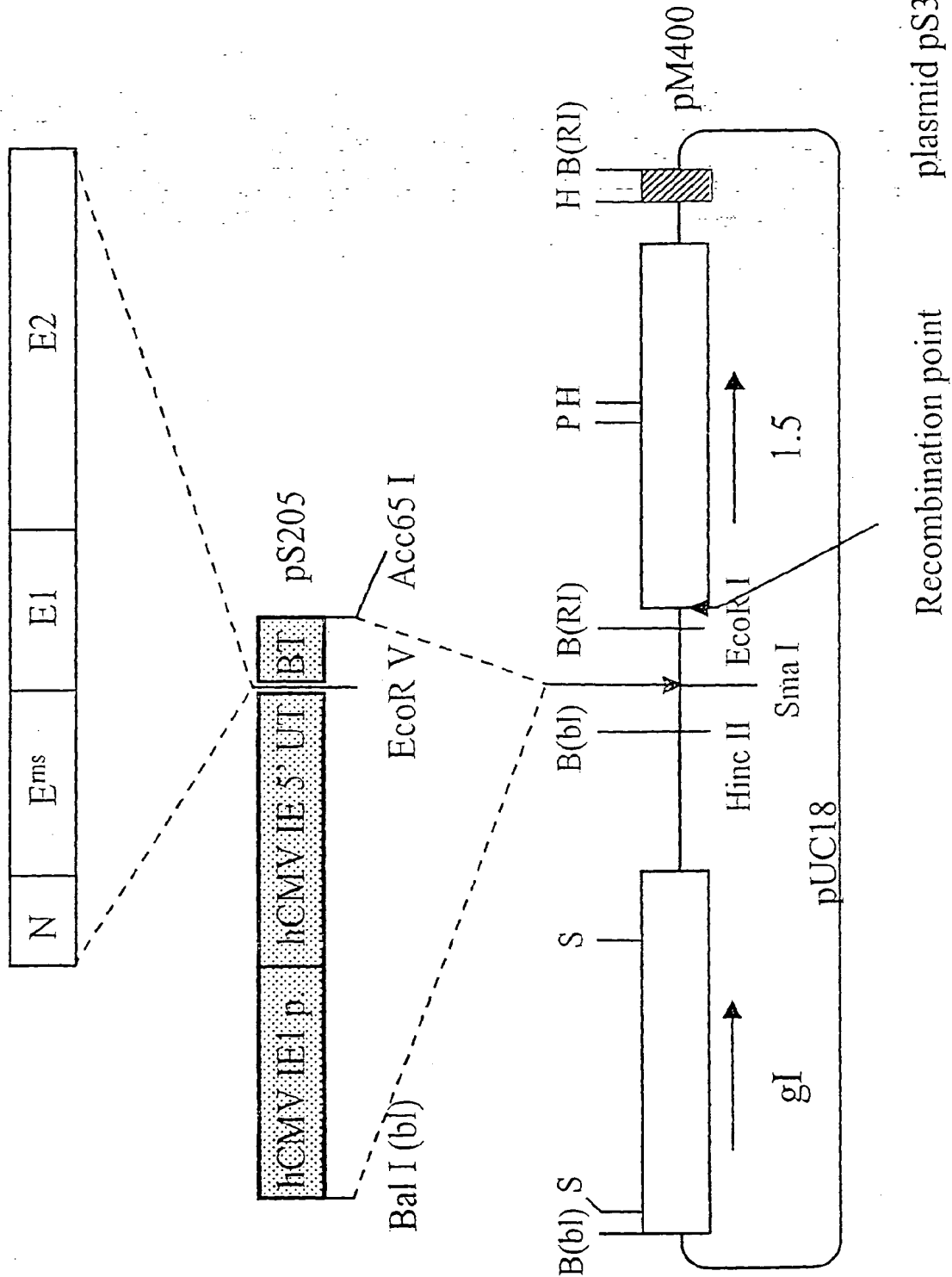
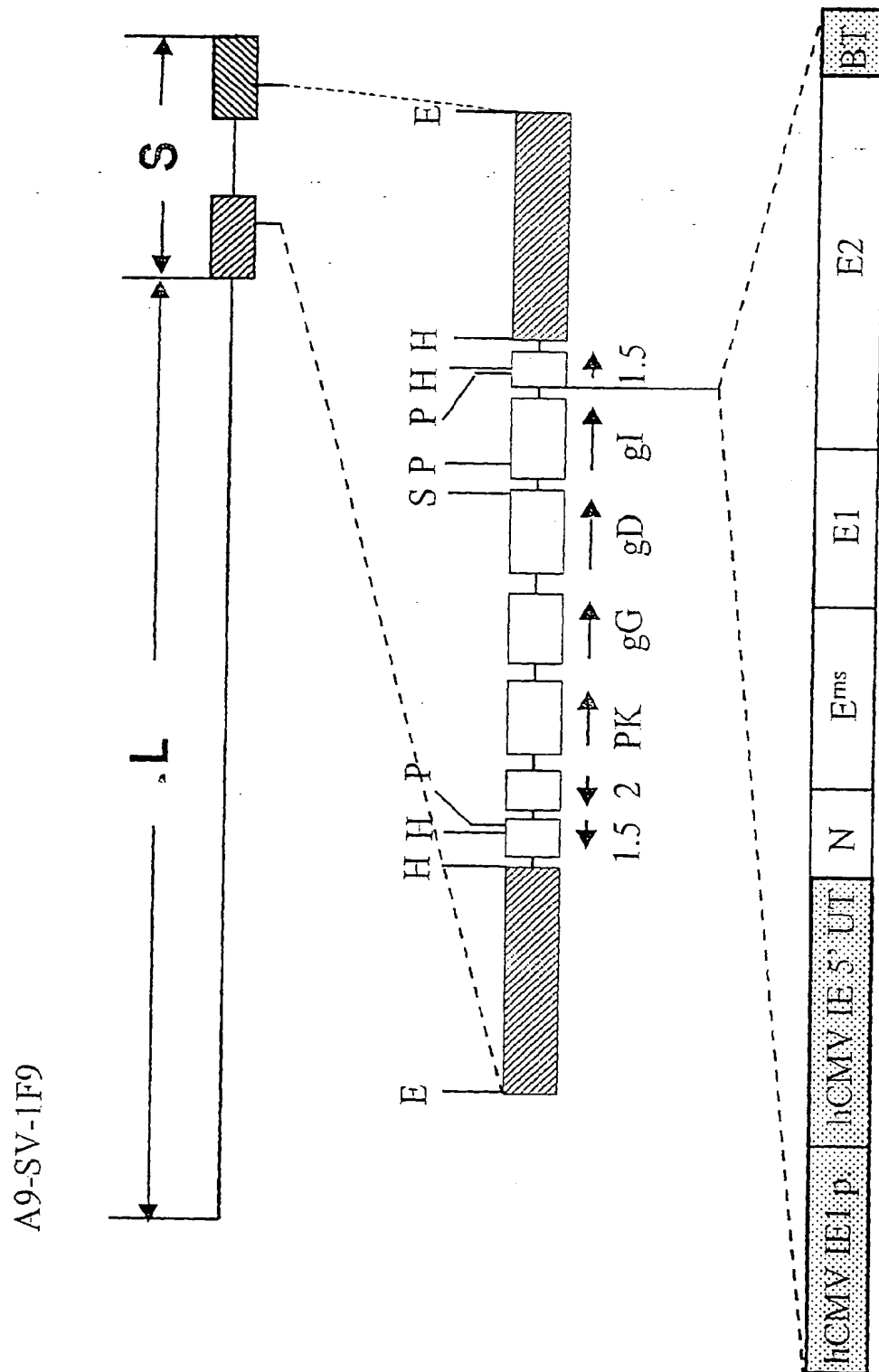
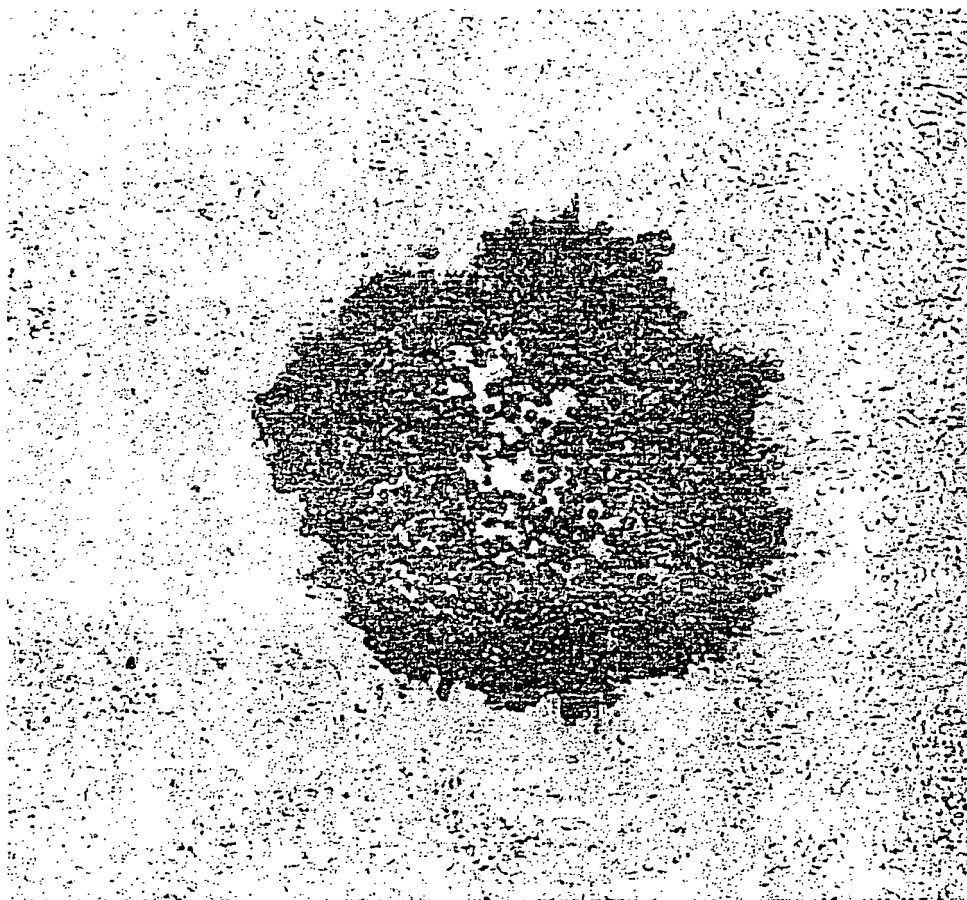


Figure 4



**Figure 5**





European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 00 11 3088

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	ELAHI SEYYED MEHDY ET AL: "Induction of humoral and cellular immune responses against the nucleocapsid of bovine viral diarrhoea virus by an adenovirus vector with an inducible promoter." VIROLOGY, vol. 261, no. 1, 15 August 1999 (1999-08-15), pages 1-7, XP002155982 ISSN: 0042-6822 * the whole document *	1-28	C12N15/40 C12N7/04 C12N15/869 A61K39/12
A	SCHMITT JUTTA ET AL: "Expression of bovine viral diarrhoea virus glycoprotein E2 by bovine herpesvirus-1 from a synthetic ORF and incorporation of E2 into recombinant virions." JOURNAL OF GENERAL VIROLOGY, vol. 80, no. 11, November 1999 (1999-11), pages 2839-2848, XP002155983 ISSN: 0022-1317 * the whole document *	1-28	
D,A	WO 95 12682 A (WARDLEY RICHARD C ;UPJOHN CO (US); HAANES ELIZABETH J (US)) 11 May 1995 (1995-05-11) * the whole document *	3-28	
A	US 6 001 613 A (VASSILEV VENTZISLAV B ET AL) 14 December 1999 (1999-12-14) * the whole document *	3-28	
The present search report has been drawn up for all claims			
Place of search <b>THE HAGUE</b>		Date of completion of the search <b>20 December 2000</b>	Examiner <b>Cupido, M</b>
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons &amp; : member of the same patent family, corresponding document</p>			

EP 00 11 3088 (P) (2000)

**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 00 11 3088

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.  
The members are as contained in the European Patent Office EDP file on  
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20-12-2000

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US 6001613 A	14-12-1999	NONE	

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82